Short Communication

T-Cell-Rich Large-B-Cell Lymphomas Contain Non-Activated CD8⁺ Cytolytic T Cells, Show Increased Tumor Cell Apoptosis, and Have Lower Bcl-2 Expression Than Diffuse Large-B-Cell Lymphomas

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The factor(s) responsible for the reduced B cell number and increased T cell infiltrate in T-cell-rich large-B-cell lymphomas (TCRBCLs) have not been well characterized. We studied 18 TCRBCLs and 12 diffuse large-B-cell lymphomas (DLBCLs) to compare the 1) predominant T cell subpopulation(s), 2) expression of cytotoxic granule proteins (TIA-1 and granzyme B), 3) level of tumor cell apoptosis (Apoptag system, Oncor, Gaithersburg, MD), and 4) expression of Ki-67 (Mib-1) and apoptosis-related proteins (fas (CD95), bcl-2, and p53). T cells in TCRBCLs and DLBCLs were predominantly CD8⁺ T cells expressing αβ T-cell receptors and TIA-1 (16 of 18 TCRBCLs with >50% TIA-1⁺ small lymphocytes) but lacking granzyme B (16 of 18 TCRBCLs with <25% granzyme B⁺ small lymphocytes). Scattered apoptotic tumor cells (confirmed with CD20 co-labeling) were present in 15 of 18 TCRBCLs, with 14 of 15 cases having <10% apoptotic cells. No apoptotic cells were seen in 12 of 12 DLBCLs. In 16 of 16 immunoreactive TCRBCLs, <25% tumor cells were bcl-2⁺, whereas 6 of 12 DLBCLs had >50% bcl-2⁺ tumor cells. CD95 (fas) expression was also lower, with 3 of 18 (16.7%) TCRBCLs *versus* 4 of 12 (33%) DLBCLs having >25% CD95⁺ tumor cells. TCRBCLs and DLBCLs had similar levels of p53 and Ki-67 (Mib-1) expression. Thus, T cells in TCRBCLs are non-activated cytotoxic T lymphocytes (TIA-1⁺, granzyme B⁻). Tumor cell apoptosis (perhaps cytotoxic T cell mediated) may partly account for the decreased number of large (neoplastic) B cells in TCRBCLs, but

other factors (ie, decreased bcl-2 expression) may also be needed. *(Am J Pathol 1998, 153:1707–1715)*

T-cell-rich large-B-cell lymphoma (TCRBCL) is a term used to describe a group of diffuse large-B-cell lymphomas with a prominent infiltrate of small T lymphocytes.[1–6] The percentage of T cells needed to classify a lymphoma as a TCRBCL is somewhat controversial, but most authors would accept tumors as TCRBCL if at least 65% to 90% of the lymphoid infiltrate is T cells, with the remainder being large B cells.[7] The B cells have been shown to be a clonal, presumably neoplastic cell population by demonstration of light chain restriction and/or by the presence of immunoglobulin gene rearrangements.[2]

Despite the abundance of T cells, most studies indicate the clinical behavior of TCRBCLs is not significantly different from other diffuse large-B-cell lymphomas (DLBCLs), which typically have few tumor-infiltrating lymphocytes (TILs). Previous studies by Chittal et al[5] and Delabie et al[6] have indicated that some TCRBCLs may follow a more aggressive clinical course. These studies examined a total of nine and six patients, respectively. Of the combined 15 patients, 6 followed a very aggressive clinical course with short survival (≤18 months). Of these six patients with an aggressive clinical course, it is interesting to note that four had been originally diagnosed and treated as Hodgkin's disease rather than non-Hodgkin's lymphoma. Therefore, the poor results in some of these patients may have been related to inappropriate initial therapy rather than due to an inherently more aggressive biological behavior. More recent studies by Greer et al[8] and Rodriguez et al[9] have examined larger series of 44

and 23 patients, respectively, and have shown no significant survival difference between TCRBCLs and DLBCLs. Finally, data examining bone marrow involvement in TCRBCLs[10] have shown no significant difference in overall 4-year survival in patients with TCRBCL involving bone marrow compared with a group of control patients with bone marrow involvement by histologically concordant DLBCL when treated with curative intent.

The few TCRBCLs analyzed by flow cytometry or frozen-section immunohistochemistry have shown a predominance of CD4^{+} T cells with a CD4:CD8 ratio compatible with a reactive infiltrate.[1,2] At least one case report with flow cytometric immunophenotyping has suggested that the predominant T cell subpopulation is CD8^{+}.[11] However, no previous studies have fully characterized the T cells based on functional and T cell receptor (TCR) framework antigen expression. Recently, a number of antibodies have been developed to allow CD4 and CD8 subtyping of T cells in fixed, paraffin-embedded tissue sections to assess the type of TCR expressed and to evaluate the presence of cytotoxic-granule-associated proteins such as TIA-1 and granzyme B.[12–14]

Furthermore, the factor(s) responsible for the increased number of T cells and/or the reduced number of neoplastic B cells in TCRBCLs have not been extensively studied. Previous studies from our group have suggested that interleukin (IL)-4 may play a role in the proliferation of T cells and/or the suppression of B cell growth.[15] However, the role of other proliferation-associated and/or apoptosis-associated proteins has not been investigated.

We therefore undertook the present study to compare TCRBCLs and DLBCLs to 1) define the predominant T cell phenotype in TCRBCLs in paraffin-embedded, fixed tissue sections, 2) to assess the expression of cytotoxic T lymphocyte (CTL)-associated proteins, namely TIA-1 and granzyme B, in the T cell infiltrate of TCRBCL and DLBCL, 3) to examine tissue sections for evidence of apoptosis in both TCRBCL and DLBCL using a commercially available terminal deoxynucleotidyl transferase (TdT) end-labeling technique (Apoptag, Oncor, Gaithersburg, MD), and 4) to examine and compare the expression of the proliferation-associated marker Ki-67 (Mib-1) and the apoptosis-associated proteins fas (CD95), bcl-2, and p53 in TCRBCL *versus* DLBCL.

Materials and Methods

Selection of Cases

Eighteen TCRBCLs and twelve DLBCLs were evaluated by paraffin immunoperoxidase staining for the antigens described below and for the presence of apoptotic tumor cells using the Apoptag *in situ* detection kit (Oncor). The clinicopathological features of the cases chosen for study have been previously published.[2,15] As defined in previous publications,[2,8,15] lymphomas were classified as TCRBCLs if the majority (ie, more than 50%) of cells were small T cells by paraffin immunoperoxidase studies for CD3 and/or CD45RO (UCHL-1). One TCRBCL (case 17 in Ref. 2) and three DLBCLs (see Ref. 15) described

previously could not be evaluated in the present study because of insufficient tissue.

Paraffin Immunoperoxidase Studies

Paraffin sections (4 μm) from B5-fixed tissues were stained by the streptavidin-biotin complex method and horseradish peroxidase (HRP) reacted with 2′,5′-diaminobenzidine (DAB) according to manufacturer's instructions or previously published modifications[12–13,16] for expression of the following antigens: CD20 (L26), CD8, CD3 (polyclonal), p53, and bcl-2, all from Dako, Carpinteria, CA; CD56 (123C3) from Zymed Corp., South San Francisco, CA; CD4 from Vector Laboratories, Burlingame, CA; TCR-$\alpha\beta$ (βF-1) and TCR-$\gamma\delta$ (TCRδ1), both from Endogen, Woburn, MA; Ki-67 (Mib-1) from Immunotech, Westbrook, ME; and TIA-1 from Coulter Immunology, Hialeah, FL.

In addition, immunoperoxidase staining for CD95 (fas) expression was performed using a rabbit polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA), with microwave epitope retrieval in 10 mmol/L sodium citrate, pH 6.0 (two 5-minute applications). Paraffin-embedded, B5-fixed sections (4 μm) were deparaffinized in 2 g% iodine in xylene (10 minutes at 25°C), followed by three washes (five minutes each at room temperature) in xylene alone. Slides were rehydrated by sequential washes (5 minutes each) in 100% ethanol (two times), 70% ethanol, and phosphate-buffered saline (PBS; 50 mmol/L sodium phosphate, 200 mmol/L sodium chloride, pH 7.4). Endogenous peroxidase activity was inactivated by incubating slides in 3% hydrogen peroxide in PBS (5 minutes at 25°C), followed by three washes in PBS (5 minutes each at 25°C). After blocking nonspecific antibody-binding sites (Protein Blocker, Research Genetics, Huntsville, AL) for 5 minutes at 25°C, slides were incubated with anti-CD95 at a 1:50 dilution in PBS for 60 minutes at 37°C and washed three times in PBS. Staining was then detected using biotinylated goat anti-rabbit antibody (Dako; 60 minutes at 37°C, followed by three washes in PBS), streptavidin-conjugated horseradish peroxidase (HRP; Research Genetics; 30 minutes at 25°C, followed by three washes in PBS), and DAB (Stable DAB, Research Genetics; two applications of 5 minutes each at 25°C). Sections were then washed three times in distilled water, stained with hematoxylin (Autohematoxylin, Research Genetics), and mounted onto coverglasses using standard techniques.

Immunoperoxidase staining for human granzyme B expression (1:20 dilution, clone GrB-7; Monosan, Uden, The Netherlands) was performed by the streptavidin-biotin complex method using HRP with DAB on B5-fixed paraffin-embedded sections (4 μm) pretreated by microwave epitope retrieval in 100 mmol/L sodium citrate, according to the manufacturer's directions.

In all immunostaining procedures, the adequacy of staining was verified with appropriate positive controls, including tonsil (CD20, CD3, CD4, CD8, βF1, TIA-1, and Mib-1), hepatosplenic $\gamma\delta$ T cell lymphoma (granzyme B, TCRδ1), retinoblastoma (CD56), follicular lymphoma (bcl-

Table 1. Immunophenotype of Small T Cells in T-Cell-Rich Large-B-Cell Lymphomas

Case	Age (Years)	Sex	TIA-1	GrB	CD4	CD8	CD56	βF1	TCRδ1
1	55	M	3+	1+	0	3+	1+	3+	1+
2	66	M	3+	1+	0	2+	1+	2+	1+
3	42	F	3+	1+	0	2–3+	1+	1+	1+
4	18	F	3+	1+	4+	1+	1+	4+	1+
5	68	F	3+	1+	0	1+	1+	2+	1+
6	74	M	3+	1+	0	4+	1+	2–3+	1+
7	44	F	3+	1+	1+	2–3+	1+	3+	1+
8	47	M	2+	1+	1+	2+	1+	3+	1+
9	69	M	3+	1+	0	2–3+	1+	3+	1+
10	56	F	4+	2+	0	4+	1+	1+	1+
11	67	F	3+	1+	1+	1+	1+	4+	1+
12	57	M	3+	1+	2+	3+	1+	4+	1+
13	52	M	4+	1+	2+	3+	1+	4+	1+
14	36	M	3+	1+	1+	4+	1+	3+	1+
15	36	F	4+	1+	0	2+	1+	4+	1+
16	18	M	1+	1+	1+	1+	1+	2–3+	1+
17	92	F	4+	1+	1+	3+	1+	2–3+	1+
18	43	M	4+	3+	1–2+	4+	1+	3+	1+

Patients 10 and 12 represent relapsed lymphomas with a TCRBCL pattern, occurring 8 and 7 years, respectively, after an initial diagnosis of DLBCL. (See also Tables 2 and 4 footnotes.) GrB, granzyme B; M, male; F, female. Results are shown based on the following scoring system: 0, no small cells marking; 1+, 1% to 25% small cells marking; 2+, 26% to 50% small cells marking; 3+, 51% to 75% small cells marking; 4+, 76% to 100% small cells marking.

2), breast carcinoma (p53), and reactive lymph node (CD95).

Apoptosis Assay

Mercury salts and paraffin were removed from sections (4 μm) of B5-fixed tissues by soaking in 2 g% iodine in xylene for 10 minutes followed by three washes (5 minutes each) in xylene only. Sections were then rehydrated and processed using the Apoptag *in situ* detection kit (Oncor) according to the manufacturer's instructions, except that tissues were counterstained with Gill's hematoxylin (Fisher Scientific, Fair Lawn, NJ). Positive control sections (from a case of HIV-related lymphadenopathy) were run with each experiment. TCRBCL cases were also double labeled for CD20 expression (L26) followed by biotinylated goat anti-mouse antibody (Dako) and a streptavidin-biotin detection system with alkaline-phosphatase and fast red substrate (Research Genetics) according to the manufacturer's instructions.

Evaluation of Staining

Antibody staining was evaluated independently and jointly by at least two pathologists (R.E. Felgar, K.R. Steward, and/or W.R. Macon). Staining of the small lymphocytes was scored as follows: 0, no staining; 1+, 1% to 25% small lymphocytes positive; 2+, 26% to 50% small lymphocytes positive; 3+, 51% to 75% small lymphocytes positive; 4+, 76% to 100% small lymphocytes positive. Staining of the large (neoplastic) lymphocytes was also evaluated using the following scale: 0, no staining; 1+, 1% to 10% large cells positive; 2+, 11% to 25% large cells positive; 3+, 26% to 50% large cells positive; 4+, >50% large cells positive.

Results

Patient Populations

The general demographic features of the patients from whom the TCRBCLs and DLBCLs were obtained for study were similar. The TCRBCL group consisted of 10 males and 8 females with a mean age of 52.2 years (median, 53.5 years); the DLBCL group consisted of 7 males and 5 females with a mean age of 58.7 years (median, 56.5 years). The age range of the TCRBCL group (18 to 92 years), however, was much broader than that seen in the DLBCL group (46 to 74 years).

Immunophenotype of Small Lymphocytes in TCRBCLs and DLBCL

Immunophenotyping data for the small lymphocytes in the TCRBCL group are summarized in Table 1. In 16 of 18 TCRBCLs, more than 50% of the TILs were TIA-1$^+$ (score of 3+ to 4+) with most of them also expressing CD8 and $\alpha\beta$ TCRs (βF1$^+$) (see Table 1 and Figure 1). One case (number 4 in Table 1) had a predominance of CD4$^+$ and βF1$^+$ TILs that were also TIA-1$^+$. Staining for granzyme B showed a few cells staining in all cases but a much lower number staining in comparison with TIA-1 (16 of 18 cases with 25% or fewer granzyme B$^+$ cells), suggesting a non-activated functional status. In most cases, few lymphocytes stained for CD4, CD56, or $\gamma\delta$ TCRs (TCRδ1$^+$). Staining of the DLBCLs showed only a few scattered small lymphocytes with a lower percentage of TIA-1$^+$ cells (9 of 12 cases with <50% small lymphocytes TIA-1$^+$) than in TCRBCLs; the percentage of small lymphocytes in DLBCLs expressing granzyme B was also generally lower in comparison with TIA-1 expression, with 8 of
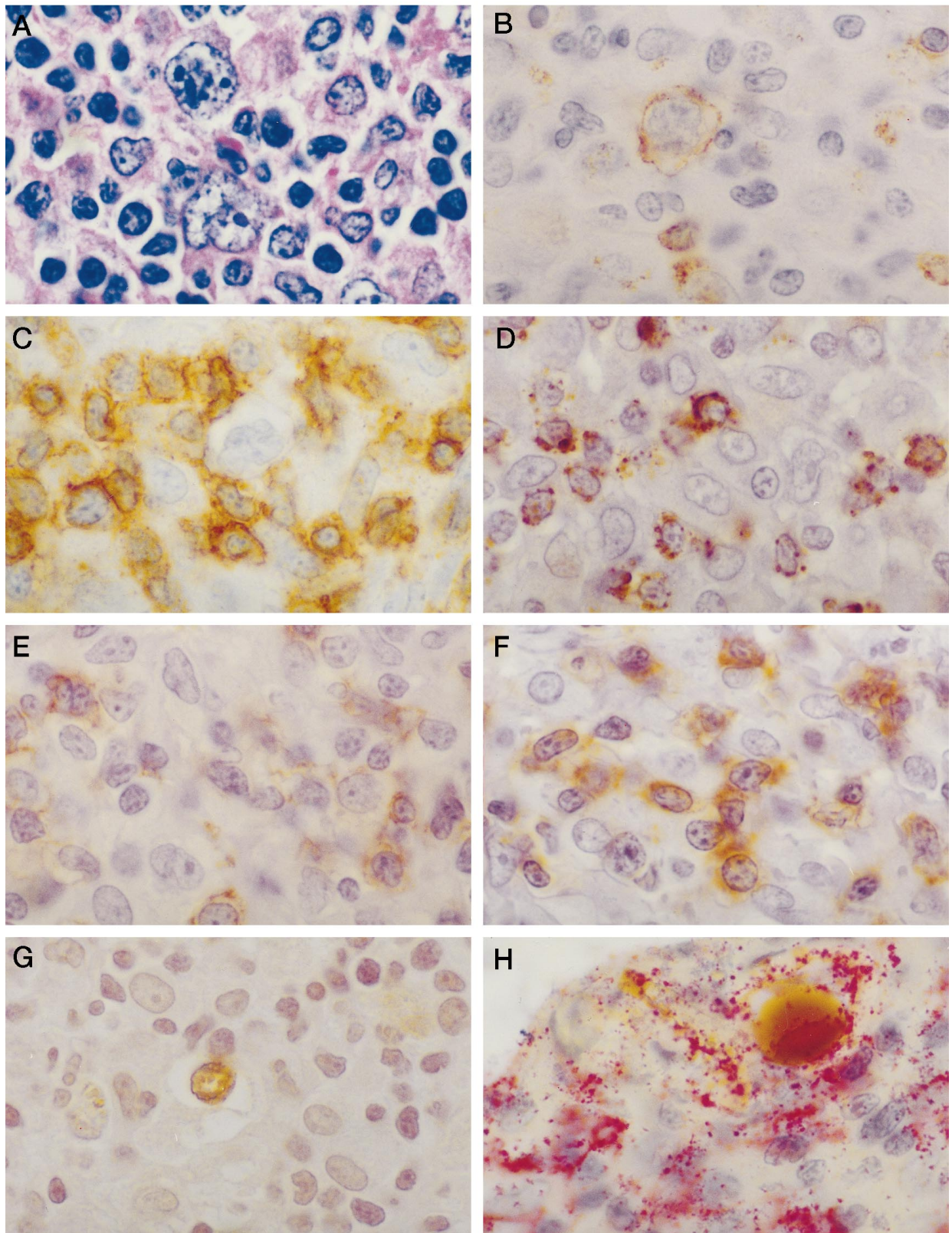
Figure 1. Staining pattern in a typical case of TCRBCL (case 15 in Tables 1 and 3). **A:** Typical H&E appearance. **B** to **F:** Immunostains for CD20 (**B;** L26), CD3 (**C**), TIA-1 (**D**), CD8 (**E**), and βF1 (**F**) demonstrating that most of the TILs are CD8$^+$ and βF1$^+$ T cells. **G:** Labeling of scattered large apoptotic cells with the Apoptag system that are also CD20$^+$ on co-labeling studies. **H:** L26 antibody with alkaline phosphatase and fast red substrate. Magnification (oil immersion), ×1000.

Table 2. Immunophenotype of Small T Cells in Diffuse Large-B-Cell Lymphomas

Case	Age (Years)	Sex	TIA-1	GrB	CD4	CD8	βF1
1	62	M	2–3+	1+	0	2–3+	NR
2	74	M	3+	1+	0	3+	3+
3	46	M	2+	2+	0	2+	2+
4	73	F	2+	1–2+	0	3+	3+
5	58	F	2+	2+	1+	3+	3+
6	57	M	1+	1+	0–1+	3+	3+
7	56	F	3+	2–3+	0	3+	3+
8	66	M	1+	0	0	3+	3+
9	56	M	3+	1+	0	3+	3+
10	52	M	1+	0	0	1–2+	3+
11	50	F	2+	1+	0	2+	2–3+
12	54	F	1+	1+	0	2+	2+

Initial neoplasm in case number 6 was a DLBCL; patient had relapsed lymphoma 7 years later with a TCRBCL pattern (case 12 in Tables 1 and 3). The initial neoplasm in case 7 also was a DLBCL, with a relapse 8 years later with a TCRBCL pattern (case 10 in Tables 1 and 3). See Table 1 for scoring system. GrB, granzyme B; M, male; F, female.

12 cases showing <25% granzyme B$^+$ cells (see Table 2). In general, most of the TILs in DLBCLs were also CD8$^+$ and βF1$^+$ (see Table 2 and Figure 2). Because most lymphocytes were CD8$^+$ and βF1$^+$, staining for CD56 and TCRδ1 was not evaluated in the DLBCLs.

Apoptosis Studies

TdT end-labeling studies using the Apoptag *in situ* detection kit highlighted the presence of apoptotic cells in 15 of 18 cases of TCRBCL (see Table 3 and Figure 1); double-labeling studies using L26 with an immunoalkaline phosphatase detection system showed that these cells were marking as CD20$^+$ B cells (ie, consistent with the neoplastic cell population; see Figure 1.) In general, the number of apoptotic cells in TCRBCLs was low, with 14 of 15 cases having <10% Apoptag-labeled cells. No apoptotic cells were seen in the DLBCL cases (Table 4 and Figure 2).

Expression of Proliferation- or Apoptosis- Associated Antigens

Data regarding the expression of the proliferation-associated antigen Ki-67 (Mib-1 staining) and apoptosis-related antigens (p53, bcl-2, and CD95/fas) in the tumor (large B) cell population are summarized in Tables 3 and 4.

TCRBCLs had a lower percentage of tumor cells expressing bcl-2 (16 of 16 immunoreactive cases had <25% bcl-2$^+$ tumor cells) than DLBCLs (4 of 12 cases with <25% bcl-2$^+$ tumor cells and 6 of 12 cases with >50% bcl-2$^+$ tumor cells). CD95 (fas) expression was slightly lower in TCRBCLs, as 3 of 18 (16.7%) cases had >25% CD95$^+$ tumor cells as compared with DLBCLs, which had 4 of 12 (33%) cases with >25% CD95$^+$ tumor cells.

In general, there were no consistent differences in the percentage of tumor cells expressing Ki-67 (Mib-1$^+$) or p53 between TCRBCLs and DLBCLs. In both TCRBCLs and DLBCLs, 83% of cases (15 of 18 TCRBCLs and 10 of 12 DLBCLs) stained positively for Mib-1 in <25% of tumor cells. There was also comparable p53 staining, as 15 of

18 (83%) TCRBCLs and 9 of 12 (75%) DLBCLs were positive in <25% of tumor cells.

Discussion

The present study was designed to address three basic issues: 1) to determine the phenotype of the T cell infiltrate in TCRBCL, with emphasis on the presence of cytotoxic-lymphocyte-associated markers, 2) to investigate the role of apoptosis, possibly CTL-mediated, in TCRBCL, and 3) to investigate possible mechanism(s) by which increased apoptosis or reduced cellular proliferation might account for the lower number of neoplastic large B cells in TCRBCL in comparison with DLBCL.

Previous flow cytometry studies of TCRBCLs, including previous analyses on material from five of the cases studied here, have suggested that the predominant T cell subpopulation is a CD4$^+$ T cell.[1–2] Conversely, at least one case report using flow cytometric immunophenotyping has suggested that the predominant small lymphocyte may be a CD8$^+$ T cell.[11] However, the number of cases studied in this manner is small, and such studies are hindered by the inability to directly correlate the flow cytometry data with morphology. In the present study, 8 of 18 TCRBCLs showed CD8 positivity in >50% of the small lymphocytes (score of 3+ or 4+; Table 1), and in an additional 3 cases, CD8$^+$ lymphocytes appeared to be the predominant T cell subset (score of 2+ to 3+; Table 1). Previous studies on peripheral T cell lymphomas by our group[13] have estimated the sensitivity of CD8 immunostaining by this paraffin immunoperoxidase (PIP) technique to be 90% using flow cytometry as the comparative standard. PIP CD4 staining, in contrast, had an estimated sensitivity of 64%. Therefore, it is possible that we have underestimated the true CD4$^+$ T cell subset in the present study. Indeed, in six cases (cases 2, 5, 9, 11, 13, and 16 in Table 1), the total percentage of CD4$^+$ and CD8$^+$ cells combined appears to be less than 75% of all T cells. However, in five of these cases (cases 2, 5, 9, 11, and 15), the percentage of TIA-1$^+$ cells was scored as 3+ or greater, indicating that the majority of cells are cytotoxic lymphocytes. In most of the cases, TIA-1 expression appeared to correlate with a CD8$^+$ CTL pheno-
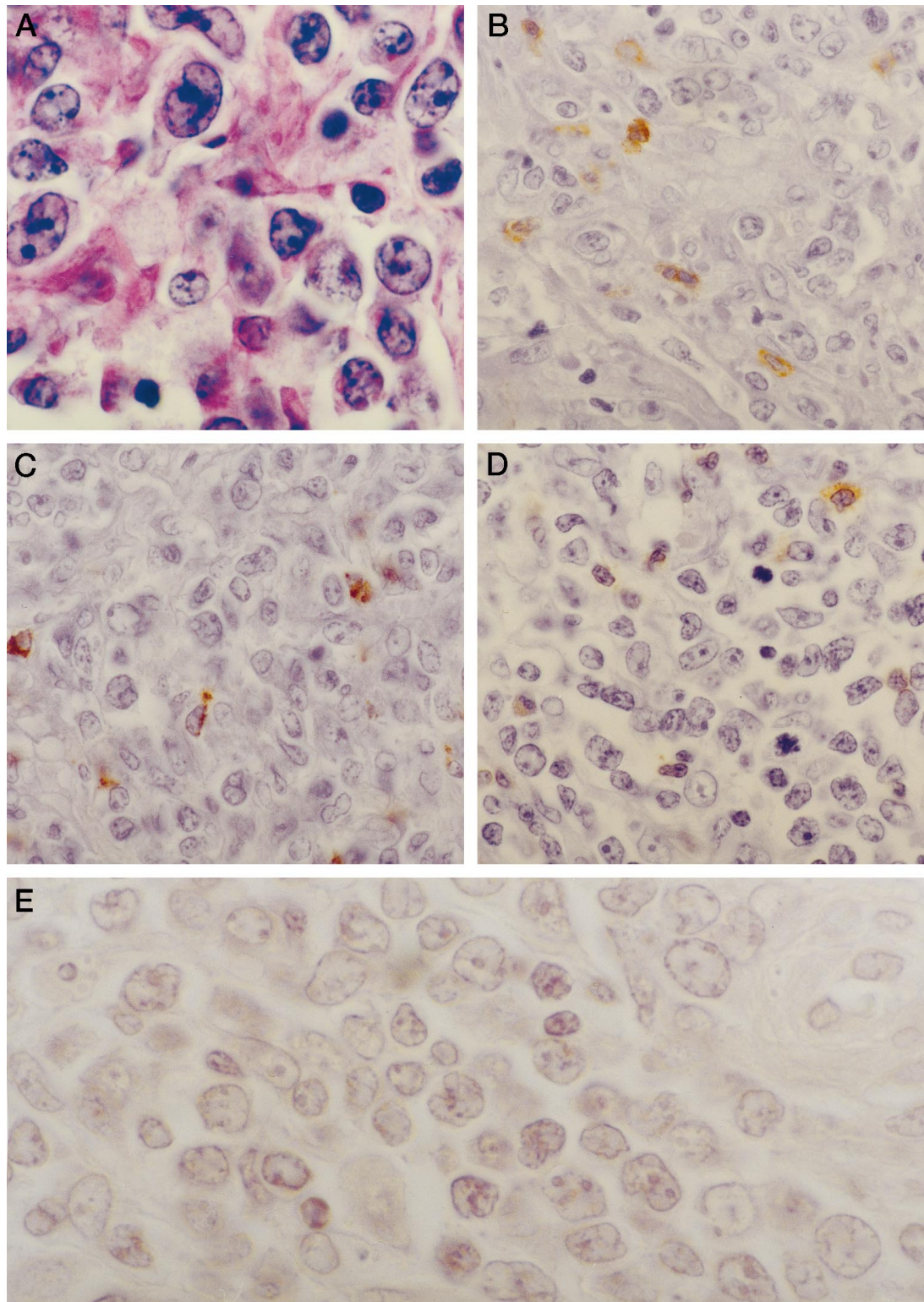
Figure 2. Staining pattern in a typical case of DLBCL (case 2 in Tables 2 and 4). **A:** Typical H&E appearance of large transformed lymphocytes. **B:** CD8 staining; **C:** TIA-1 staining; **D:** βF1 staining, showing that a few TILs are present that are also CD8$^+$ and βF1$^+$ T cells. **E:** No apoptotic tumor cells are detectable with the Apoptag assay system. Magnification (oil immersion), ×1000 (**A** and **E**) and ×600 (**B** to **D**).

type; it therefore seems probable that the true number of CD8$^+$ T cells was underestimated in these five cases. Furthermore, in eight cases (Table 1), no CD4$^+$ cells

were detected within the tumor. However, in nearly all of these cases, CD4$^+$ cells were seen within adjacent lymphoid tissues not involved by tumor; so it is not likely that

Table 3. Immunophenotype of Large (Neoplastic) B Cells in T-Cell-Rich Large-B-Cell Lymphomas

Case	Age (Years)	Sex	Apoptag	p53	Bcl-2	Mib-1 (Ki-67)	CD95 (fas)
1	55	M	1+	0	NR	1+	3+
2	66	M	0	1+	1+	1+	3+
3	42	F	1+	1+	0	1+	2+
4	18	F	2+	3+	0	2–3+	1+
5	68	F	1+	1–2+	0	2+	1+
6	74	M	0	1+	1+	1+	1+
7	44	F	1+	3+	0	4+	3+
8	47	M	1–2+	1+	0	2+	1+
9	69	M	1+	0	NR	1+	1+
10	56	F	1+	0	0	1+	1+
11	67	F	0	1+	0	1+	1+
12	57	M	1+	3+	0	2+	1+
13	52	M	1+	0	1+	3+	1+
14	36	M	1+	2+	0	1+	1+
15	36	F	1+	1+	0	1+	1+
16	18	M	1+	1+	2+	4+	0
17	92	F	1+	1+	1+	1+	1+
18	43	M	1+	1+	1+	2+	1+

Patients 10 and 12 represent relapsed lymphomas with a TCRBCL pattern, occurring 8 and 7 years, respectively, after an initial diagnosis of DLBCL. (See also Tables 2 and 4 footnotes.) Results are shown based on the following scoring system: 0, no large cells marking; 1+, 1% to 10% large cells marking; 2+, 11% to 25% large cells marking; 3+, 26% to 50% large cells marking; 4+, >51% large cells marking. NR, tissue nonreactive with antibody (confirmed on repeat staining); M, male; F, female.

these results are solely attributable to a lack of tissue immunoreactivity. In toto, therefore, these results indicate that most of the infiltrating small lymphocytes are CD8$^+$ T cells that express $\alpha\beta$ TCR (ie, βF1$^+$) as well as the cytotoxic lymphocyte marker TIA-1.

TIA-1 (T-cell intracellular antigen-1) is a 15-kd cytolytic-granule-associated protein[17,18] expressed in both cytotoxic T cells and natural killer (NK) cells[18] and is known to cause apoptosis in target cells.[19] TIA-1 is probably identical to GMP-17 (granule membrane protein-17).[20] In most cases (16 of 18), TIA-1 expression was detected in >50% of the T cell infiltrate in TCRBCL (Table 1). In contrast, granzyme B was expressed in a much lower percentage of cells in the majority of TCRBCLs, with 16 of 18 cases having <25% of small lymphocytes expressing granzyme B. A few scattered TILs were also seen in the DLBCLs studied. These infiltrating T cells in DLBCLs also marked as CD8$^+$ CTLs with expression of $\alpha\beta$ TCR and TIA-1 and a lower level of granzyme B. TIA-1 has been shown to be expressed in both activated and non-activated cytolytic lymphocytes,[17,18] whereas granzyme B is seen predominantly in activated cytolytic cells.[21] These data would suggest that the lymphocytic infiltrate in TCRBCLs consists predominantly of non-activated CTLs, which may be only partially effective at mediating a host antitumor response. Furthermore, it indicates that although TILs in DLBCL are fewer in number, they are phenotypically similar to those seen in TCRBCL.

CTLs mediate target cell death by a complex series of events, usually resulting in an apoptotic target cell death, with DNA cleavage into 200-bp oligomers, nuclear disintegration, and finally cell lysis.[22–24] Because CTLs mediate target cell death by apoptosis, we investigated the possibility that apoptosis might be occurring within the tumor cells of TCRBCL and, if so, whether or not it might account for the decreased tumor cell number in TCRBCLs. A number of techniques have been developed to label fragmented nuclear DNA in tissue sections, including *in situ* end labeling (using the Klenow fragment of DNA polymerase I to incorporate biotin- or digitonin-tagged nucleotides into nicked DNA strands)[25] and so-called TUNEL assays (which use TdT to add tagged

Table 4. Immunophenotype of Large (Neoplastic) B Cells in Diffuse Large-B-Cell Lymphomas

Case	Age (Years)	Sex	Apoptag	p53	Bcl-2	Mib-1 (Ki-67)	CD95 (fas)
1	62	M	0	2+	4+	4+	0
2	74	M	0	1+	3+	2–3+	4+
3	46	M	0	1+	4+	1+	1+
4	73	F	0	4+	0	1+	3+
5	58	F	0	1+	4+	1+	0
6	57	M	0	2+	0	2+	4+
7	56	F	0	1+	1+	1+	0
8	66	M	0	2+	3+	3+	1+
9	56	M	0	1+	4+	2+	0
10	52	M	0	1+	4+	1+	0
11	50	F	0	4+	1+	2+	3+
12	54	F	0	3+	4+	2+	0

Initial neoplasm in case 6 was a DLBCL; patient had relapsed lymphoma 7 years later with a TCRBCL pattern (case 12 in Tables 1 and 3). The initial neoplasm in case 7 also was a DLBCL, with a relapse 8 years later with a TCRBCL pattern (case 10 in Tables 1 and 3). See Table 3 for scoring system. M, male; F, female.

nucleotides to the terminal ends of fragmented DNA strands).[26] All of the above techniques will label fragmented DNA from causes other than apoptosis, and correlation with histology is necessary to prevent misinterpretation.[27] The advantage of these methods is that they allow one to detect early apoptotic cells at a stage not obvious by routine histology. A variation on the TUNEL technique is currently available as the Apoptag kit (Oncor), which attaches digitonin-tagged nucleotides to the ends of fragmented DNA strands and detects them with an HRP-antidigitonin antibody (Fab fragment, sheep polyclonal) and DAB substrate. Previous studies[28] using tissues in which apoptosis is the known primary mechanism of cellular death have indicated that this technique is more specific for detecting apoptotic cells.

In the TCRBCLs studied, a small number of apoptotic tumor cells (confirmed by double labeling for CD20) was seen in the majority (15 of 18) of cases using the Apoptag detection kit (see Table 3 and Figure 1). No apoptotic cells were detected in the DLBCLs studied (Table 4). Although CTLs mediate cellular destruction by inducing apoptosis in target cells,[22–24] the low percentage of apoptotic cells (<10% of large B cells in most cases) observed here suggests that this mechanism is only a partial factor in accounting for the decreased number of tumor cells in TCRBCLs.

Our data are consistent with several previous observations regarding TILs in B-cell non-Hodgkin's lymphomas (NHLs). Diaz et al[29] studied B-cell NHLs not meeting the histological criteria for TCRBCL and showed an increased number of TIA-1^{+} TILs per unit area in intermediate- and high-grade NHLs in comparison with low-grade lymphomas and reactive lymphoid hyperplasias, suggesting an ineffective host antitumor response in DLBCLs. Additional studies demonstrated an apparent tyrosine phosphorylation and lck signaling defect in the TCR-CD3 complex within the T cells from patients with B-cell NHLs.[30] Our data on TCRBCLs further support the concept that TILs in intermediate- and high-grade NHLs are probably only partially effective at destroying the neoplastic B cell population and have a phenotype compatible with non-activated CTLs (TIA-1^{+}, granzyme B[−]).

Other proteins known to be involved in the mediation of apoptosis include the fas (CD95)/fas ligand (CD95L) pathway and the oncoprotein p53.[31–34] CD95 expression has been shown in approximately one-third of DLBCLs.[35] We confirmed this finding and also showed a lower level of CD95 expression in TCRBCLs compared with DLBCLs with 3 of 18 (16.7%) TCRBCLs and 4 of 12 (33%) DLBCLs containing >25% CD95^{+} tumor cells. Thus, it is unlikely that CD95-induced apoptosis plays a significant role in explaining the difference in tumor cell number in TCRBCL as compared with DLBCL. Moreover, if at least some of the apoptosis present is the result of CTL-mediated cellular lysis, then CD95 might not be expected to play a prominent role. Most CD8^{+} CTLs destroy target cells via granule-mediated exocytosis and do not rely heavily on the fas/fas ligand pathway. The CD95/CD95L pathway appears to be more important in CD4^{+} CTL-mediated killing.[36,37]

The wild-type p53 protein is known to be an important mediator of apoptosis in cells with irreparable DNA damage and is transiently overexpressed within DNA-damaged cells before apoptosis.[34] Mutations that inactivate the function of this gene and result in its overexpression are probably important in the oncogenesis of a number of malignancies.[38] Overexpression detected by paraffin immunoperoxidase methodologies are generally believed to be the result of nonfunctional gene mutations, resulting in a stable protein product.[39] Our data suggest that p53 is not likely an important mediator of either apoptosis or oncogenesis in TCRBCL or DLBCL, as the majority of each (80% of TCRBCLs and 75% DLBCLs) had detectable p53 expression in <25% of tumor cells.

An alternative explanation for the decreased number of tumor cells in TCRBCL in comparison with DLBCL is that the inhibition of apoptosis in DLBCLs allows for the accumulation of an increased number of neoplastic cells. Two findings support this hypothesis. First, no difference in proliferative activity was observed between TCRBCLs and DLBCLs as assessed by Mib-1 staining. Antibodies to Mib-1 recognize an epitope of the Ki-67 antigen that is preserved in formalin-fixed, paraffin-embedded tissues and is expressed in cells undergoing DNA replication or cellular division.[40] In both TCRBCLs and DLBCLs, 83% of cases contained <25% Mib-1^{+} tumor cells. Second, a higher percentage of DLBCLs stained positively for the bcl-2 protein (6 of 12 cases with >50% bcl-2^{+} tumor cells). In contrast 16 of 16 immunoreactive TCRBCLs had <25% bcl-2^{+} tumor cells. The protein product of the bcl-2 gene (located on chromosome 18) is known to inhibit apoptosis.[41,42] Overexpression of this protein as a result of translocation is well known to play a role in lymphomagenesis in more than 85% of follicular lymphomas, primarily those of low grade.[42–45] These data suggest that increased bcl-2 protein expression in DLBCLs may also play a role in the increased number of tumor cells in DLBCLs in comparison with TCRBCLs. Previous studies[2] by Southern blot analysis for the major and minor breakpoints of the t(14;18) translocation showed only 1 of 11 of these TCRBCLs had bcl-2 gene rearrangements, further supporting this hypothesis.

Thus, the T cell infiltrate in TCRBCLs is composed primarily of CD8^{+} CTLs, most of which have a non-activated phenotype (TIA-1^{+}, granzyme B^{−}). These data, along with the finding of apoptotic tumor cells in TCRBCLs, suggest that CTL-mediated lysis may play some role in explaining the reduced number of tumor (neoplastic B) cells in TCRBCL but probably does not play a major role. Other factors, such as increased bcl-2 expression in DLBCLs, may be more significant by providing resistance to apoptosis, thereby allowing more tumor cells to accumulate in DLBCLs. Previous studies have indicated that certain cytokines (principally IL-4) are expressed at increased levels in the neoplastic B cells of TCRBCLs.[15] It may be that local cytokine release results in increased CTL proliferation but that such cells are only partially effective at controlling tumor cell growth.

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